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Developing gene-tagged molecular marker for functional analysis of *OsZIP10* metal transporter gene in rice

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Abstract

The physiological, genetic and molecular mechanisms of metal homeostasis related genes need to be characterized from a bio-fortification perspective and should be well integrated with the breeding processes. Out of 43 genes related to metal homeostasis, transcript of *OsZIP10* gene found to be associated with grain zinc content in rice. *OsZIP10* gene belongs to *ZIP* family and is identified to encode a plasma membrane-localized zinc transporter in rice. In the present study, through allele mining identified a 10 bp insertion/deletion (*InDel*) polymorphism in the 5'upstream region of the gene. Targeting the *InDel* region five gene tagged markers were developed, named ZP1, ZP2, ZP3, ZP4, ZP5. The markers were validated in a set of selected rice genotypes contrasting for grain zinc content. Single marker analysis for allelic variation revealed significant association of markers with the phenotypic data (R^2 value 0.39). The gene-tagged markers developed in the present study would be useful in breeding for enhanced grain zinc content in rice.

Key words: Gene tagged marker, homeostasis, *InDel*, marker-aided selection, zinc

Introduction

Bio-fortification of crop especially rice is becoming a reality with the release of Zn rich rice varieties in India. The understanding of physiological, genetic and molecular basis of micronutrient related Zinc uptake, translocation, and loading into grain are still major bottle-neck in breeding micronutrient rice rich varieties. The accumulation of mineral nutrients in crops is regulated through complex cellular and genetic mechanism. The proportionate quantity of zinc present in edible part is a complex quantitative trait and governed by many major genes/QTLs (Blair et al. 2009; Diapari et al. 2014). Several genetic studies have been carried out to identify QTLs from random as well as candidate gene specific markers for grain Zn content in rice (Chandel et al. 2011; Anuradha et al. 2012). So far, tight linkage of marker with grain zinc content is still a question. Therefore, development of molecular markers based on allelic variation for low phenotypic variance trait would be a valuable contribution to improve grain micronutrient content in crops through allele mining approach.

The study is the advancement over our previous published work (Banerjee and Chandel 2011; Maurya et al. 2015), in which expression studies was performed through in-situ and RT PCR approach in different tissue types at different stages (maximum tillering stage and mid-grain filling stage). We also studied the association between the gene expression pattern and micronutrient level in tissues (flag leaf, second leaf, stem, immature grains) as well mature grains. It was observed that transcript of *OsZIP10* gene to be associated with zinc content in rice in different tissues. In order to make use of above data to establish functional relevance in marker development, *OsZIP10* gene was selected. *OsZIP10* gene belongs to the *ZIP* family and is identified to encode a plasma membrane-localized zinc transporter in rice (Ramesh et al. 2003; Ishimaru et al. 2007, 2011). *ZIP* proteins have eight predicted transmembrane-domain III and IV, containing a postulated metal ion binding site rich in conserved histidine residues (Eng et al. 1998; Guerinot 2000).

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The availability of the rice genome sequences and bioinformatic tools enhance the applicability of the allele mining strategy. Allele mining is a strategy of identification of nucleotide variations responsible for target trait and aids the development of marker specific to functional allele of the target gene (Kumar et al. 2012). Initial studies of allele mining have focused only on the identification of SNPs/InDels at coding sequences or exons of the gene, since these variations were expected to affect the phenotype of the plant. However, recent reports indicate that the variation at the upstream region, introns and 3'UTR of the gene also have significant effect on transcript synthesis and accumulation which in turn alter the trait expression (Kumar et al. 2012; Lingle and Dyre 2004). By identifying molecular markers linked to loci determining variation for micronutrients, we can select favorable genotypes without having to determine mineral levels in field conditions. Thus, the objective of current study is to develop gene tagged marker for grain zinc content in rice from 5' upstream region of *OsZIP10* gene that can be further utilized for marker assisted selection.

Materials and methods

Research material comprised of four rice genotypes with two high and two low polished grain zinc content for identification of nucleotide sequence level allelic variation in 1kb upstream of *OsZIP10* gene (Table 1).

Table 1. List of rice varieties selected for the study in terms of allelic variation in 5' upstream region of *OsZIP10* genes and their accession numbers

S.No.	Genotypes	Mean grain Zn content (Milled) (µg/gm)	Gene bank accession No.
1	Chittimuthyalu	29.1±0.1	KS697762
2	Kharigilas	28±0.8	KS697763
3	GP-145-45	13±1.3	KS697764
4	Kalimai	16±0.6	KS697765

#rice genotypes were selected based on the grain Zn contents analysed by atomic absorption spectrophotometer (AAS 200, Perkin elmer) IGKV, raipur, 2015

Genomic DNA was extracted from 3 week old seedlings of rice cultivars by modified CTAB method (Sambrook et al. 1989). Extraction buffer (100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl and 2% CTAB (w/v) was used for DNA extraction followed by chloroform: isoamyl alcohol extraction. DNA was precipitated by adding two volumes of chilled ethanol and was stored at –20^{o}C overnight. It was then centrifuged for 10 min at 10,000 rpm. The supernatant was decanted and DNA pellet was washed with 70 per cent alcohol and air-dried. The genomic DNA was resuspended in 100 µl of TE buffer (Tris 10 mM and EDTA 1.0 mM).

Using available gene sequence information of *OsZIP10* gene at Rice genome Annotation Project (rice.plantbiology.msu.edu), primer pairs were designed using the online tool, Batch primer3.0.(probes.pw.usda.gov/batchprimer3/), to amplify the 1 kb upstream region. The primer sequence was forward 5' to 3' CTTGGCATCATGTTGGTGTT (ZIP10up-F) and Reverse 5' to 3' CTCAGGACTCAGGAGCAGAA (ZIP10up-R). The annealing temperature was set to 58ºC and expected product size was 804 bp. This primer was optimized for PCR and the upstream region amplified from the selected rice genotype using a high fidelity Taq DNA polymerase (Phusion). The optimal PCR conditions used for amplification comprised 35 cycles, one for initial denaturation at 98^{o}C for 3 min and 35 cycles of denaturation at 98^{o}C for 15 s, annealing at 58^{o}C for 45 s, pre-extension at 72^{o}C for 30 s, with the final extension at 72^{o}C for 10 min. PCR product was visualized on 2% agarose gel. The amplicon bands were purified from the gel using GenElute Gel Extraction Kit (Qiagen). Purified amplicons were sequenced by Sanger sequencing technology where double pass sequencing was performed using BigDye® Terminator Cycle Sequencing (Life technologies/ Invitrogen sequencing services). Sequences obtained were aligned using the online sequence alignment tool ClustalW (www.ebi.ac.uk/Tools/clustralw/) for the identification of variation among these sequences if any, with reference to Nipponbare.

Further, primers were developed targeting a 10 bp insertion/deletion (*InDel*) polymorphism which was observed between the low grain zinc and high grain zinc rice allelic sequences. The primers (Table 2) were standardized for PCR and performed using 1 U of Taq DNA polymerase and 5x PCR buffer (Phusion) in 20µl reaction volume with a thermal profile of 98^{o}C for 3 min (initial denaturation), followed by 30 cycles of denaturation at 98^{o}C for 15 s, annealing at 61^{o}C and 62^{o}C for 45s, extension at 72^{o}C for 30s and a final extension of 72^{o}C for 1 min. The PCR products were detected using 6% polyacrylamide gels and visualized by ethidium bromide staining. Further, validation of

Table 2. Primer sequences of the gene (*OsZIP10*) based markers

S.No.	Primers	Sequences (5'-3')	Tm (^{0}C)	Expected product size (bp) (low zinc rice/high zinc rice)
1	***ZP1***	**F**:CTGCTACGAAA CGGATTCAA **R**:CAGAGAGA GGGAGGAAGGAA	62	296 /286
2	***ZP2***	**F**:TCTGCTACGAAA CGGATTCA **R**:CAGAGAGAGGG AGGAAGGAA	62	297/287
3	***ZP3***	**F**:GACTCCCAAA TTTTCCCAAC **R**:CAGAGAGGATG TCGTCATGG	61	193/183
4	***ZP4***	**F**:CTGCTACGAAA CGGATTCAA **R**:CAGAGAGGATG TCGTCATGG	60	219/209
5	***ZP5***	**F**:GGCCATCTCTGC TACGAAAC **R**:CAGAGAGGATG TCGTCATGG	61	227/217

the marker was done in a panel of selected 68 diverse rice genotypes on the basis of high and low polished grain zinc content. Further, we have scored the allelic variations as 1 or 2, for high allelic pattern and low allelic pattern respectively. These scores were then used for analysis using SPSS 16.0 (SPSS Inc.) for associating these allelic variations with the phenotypic variation for grain Zn content.

Results and discussion

Based on contrasting phenotyping data, two each of high grain zinc content and low grain zinc content rice genotypes were selected to analyze the allelic variation in upstream region of *OsZIP10* gene. PCR amplification of upstream region of *OsZIP10* showed monomorphism among rice genotypes on 2% agarose gel (Fig. 1). To dissect the information from the monomorphic bands, amplified products were further subjected to sequencing to determine variants. After sequencing, nucleotides of the four genotypes were aligned by using multiple sequence alignment tool ClustalW (www.ebi.ac.uk/Tools/clustralw/) for the

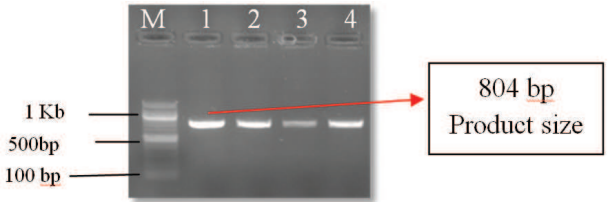

Fig. 1. PCR amplification of upstream region of *OsZIP10* gene in 4 rice genotypes; Legends M: 100 bp Ladder, 1:Chittimuthyalu, 2: Kharigilas, 3:GP-145-45, 4:Kalimai

identification of variation among these sequences. The nucleotide sequences of upstream region of *OsZIP10* gene (retrieved from Rice Genome Annotation Project) were used as reference for the comparison *i.e.*, Nipponbare. Sequencing results revealed the presence of one 10-bp *InDEL* and 2 single nucleotide polymorphisms (SNPs) as common polymorphisms between the low grain zinc content and high grain zinc content rice genotypes. Among these, a 10-bp *InDEL* was conspicuous (Fig. 2). Sequence obtained by

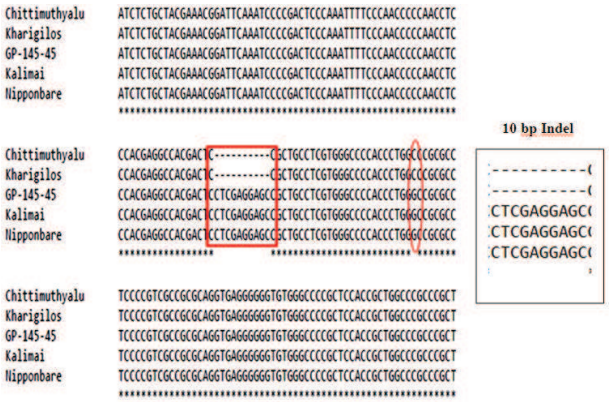

Fig. 2. Multiple sequence alignment showing result for *OsZIP10* 5' upstream sequences of different rice genotypes with reference to Nipponbare indicating that 10 bp insertion/ deletion is present in low/high grain zinc rice genotypes

sequencing of amplicons of four rice genotypes was submitted to gene bank (NCBI) with Accession No. mentioned in Table 1.

The sequence divergences were used to develop gene tagged markers by designing flanking primers and then amplifying the panel of high and low grain zinc content rice genotypes. Five primers were designed targeting the 10 bp *InDel* region, in such a way that the 10 bp deletion in high grain Zn and 10 bp

insertion in low grain Zn rice genotypes would be observed. PCR products was subjected to electrophoresis on a 6% polyacrylamide gel and visually examined after ethidium bromide staining (Table 2). Only those primer pairs which gave rise to unambiguous polymorphic PCR products could be converted into gene tagged marker. All the five pair of allele specific primers was optimized and banding pattern was observed in the set 4 rice genotypes whose sequencing was performed (Fig. 3). It was observed

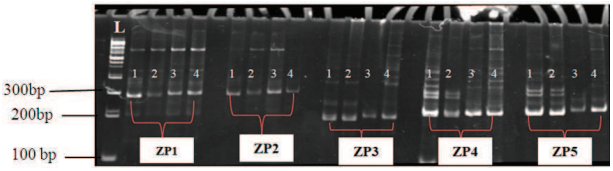

Fig. 3. PCR amplification of gene tagged markers among 4 rice genotypes. Gene tagged markers ZP1, ZP2, ZP3, ZP4 and ZP5 showing 286bp, 287bp, 183bp, 209bp, 217bp amplicon for high zinc lines (1. Chittimuthyalu and 2. Kharigilas) and 296bp, 297bp, 193bp, 219bp and 227bp amplicon for low zinc lines (3. GP145-45 & 4. Kalimai) in 6% PAGE

Table 3. List of rice genotypes used to validate the gene tagged markers

S.No.	Rice genotypes	Allelic variations	Mean grain Zn content (milled) (µg/gm)[#]
1	Atma sheetal	1	15.6
2	Bada gada khuta	1	18.2
3	BAM 4168	1	19.5
4	BAM 811	2	29.3
5	Bamleshwari	1	16.4
6	Banda	1	13.9
7	Bangla gurmatia	2	25.4
8	Banspatri	1	15.6
9	Bhainsa puchi	1	19.3
10	Bhata makdo	1	17.5
11	Bhathaili gurmatia	2	26.3
12	Botki gurmatia	2	32.8
13	Chakari gurmatia	2	29.8
14	Chandrahasini	1	23.4
15	Chapti gurmatia	1	18.3
16	Chepti gurmatia	1	17.6
17	Chinnor	2	30.0
18	Chitimutaliya	2	31.1
19	Cure1	1	18.8
20	Danteshwari	1	20.7
21	Deshi gurmatia	1	19.7
22	Doukong	1	19.0
23	Dubraj	1	20.2
24	Farsha phool	2	24.2
25	Gang puriha	1	20.9
26	GP145-29	2	29.7
27	GP145-45	1	13.0
28	Gurmatia	2	25.8
29	HMT	1	21.0
30	IR64	1	19.5
31	Jal ponga	2	25.0
32	Jhunki gurmatia	2	24.9
33	Kalimai	1	16.1
34	Kharigilas	2	30.8
35	Kasturi	1	17.0
36	Mo.5	1	17.4
37	Nunki gurmatia	1	22.7
38	Pana	1	19.6
39	Pandari gurmatia	2	34.7
40	Parau gurmatia	2	33.9
41	R-2270	1	32.2
42	R5	1	30.9
43	Rani kajar	2	27.3
44	RP-2333	1	13.2
45	Samund chini	1	14.9
46	Sheetal bhog	1	18.6
47	Shuklaphool	2	19.4
48	Srikamal	1	26.1
49	Swarna	1	16.6
50	Tilaksturi	2	31.1
51	Tulsiprasad	1	30.1
52	Vishnu bhog	2	27.5
53	IET25470	2	24.0
54	IET25475	1	23.1
55	IET25446	1	27.5
56	IET25461	1	25.0
57	R-RHZ-LI22	1	26.6
58	IET25477	1	23.3
59	Nipponbare	1	17.0
60	MTU1010	1	19.3
61	IR94033-1	1	24.7
62	IR681444	1	25.2
63	Kalam gurmatia	2	30.3
64	Chir 8	2	28.0
65	Lalmati	2	25.7
66	Basmati 370	2	22.5
67	Bisni	1	28.0
68	Morobarakan	1	17.0

*scoring of allelic variation resulted in genotypic data as 1 or 2. 1 indicates allele with low grain Zn content and 2 indicates the allele with high grain zinc content

that all five primers were showing the bands as per expected size on the basis of marker designed targeting InDel region (Table 2). For instance, PCR amplification of ZP3 marker showing 183bp and 193bp amplicons in high zinc rice (Chittimuthyalu, Kharigilas) and low zinc rice (Kalimai, GP-145-45) respectively. Further, to check the utility of newly developed gene tagged markers *viz*., ZP1, ZP2, ZP3, ZP4, ZP5, we have validated markers in the panel of 68 diverse rice genotypes selected on the basis grain zinc content (Table 3). All 5 primers showed the same allelic pattern distinguishing in its product size and have polymorphism as per assumed with few exceptions. Among 68 rice genotypes, 44 and 24 genotypes displayed amplification of low grain zinc content and high grain zinc content, respectively with respect to the gene tagged markers. For instance, the low zinc rice genotypes, Swarna, Atma sheetal, IR64, Bamleshwari, Samundchini, Sheetal bhog showed a low grain zinc allelic pattern (allele 1) and the high zinc rice genotypes, BAM811, Bangla gurmatia, GP-145-49, Ranikajar, Vishnu bhog showed a high grain zinc allelic pattern (allele 2) with all five gene tagged markers ZP1, ZP2, ZP3, ZP4 and ZP5 (Fig. 4).

SMA was done using SPSS 16 (SPSS Inc.) software to study the contribution of markers in the set of diverse rice genotypes. Newly developed markers manifested significant association with the grain zinc content at 1% level of significance. Phenotypic variance exhibited by allelic variations for grain zinc content in polished rice is 39% (P value:7.6E-09**, R^2 value: 0.39). These results illustrate that nucleotide changes in the upstream regions of the gene can lead to significant changes in the phenotype, which is valuable for a breeder to improve the low phenotypic variance trait.

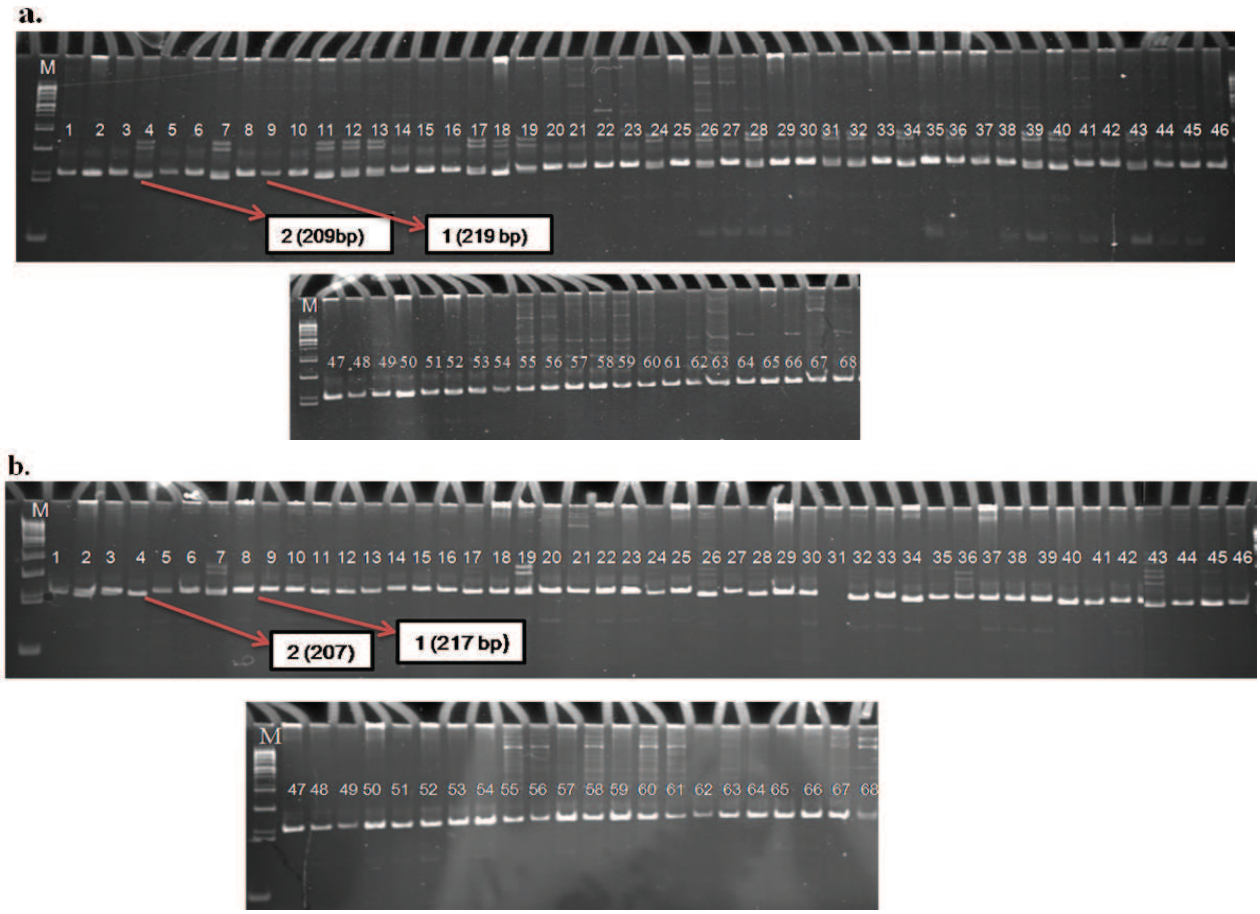

Fig. 4. Validation of gene tagged markers in rice. Gene tagged DNA marked markers showing 209 bp and 207bp amplicon in high grain Zn containing rice and 219 bp and 217 bp amplicon in low grain Zn content rice. Legends. M = Marker, a = PAGE gel profiling of rice genotypes with ZP4 marker, b = PAGE gel profiling of rice genotypes with ZP5 marker

Due to rapid accumulation of sequence and expression data in databases lead to the discovery and annotation of new genes which would enable the development of allele-specific markers (Spooner et al. 2005). Since, it is well known that promoter elements play a key role in gene regulation and any changes in their sequences will also influence gene expression resulting in variable trait expression (Ramkumar et al. 2014). Therefore, in the present study we have targeted the upstream sequence of *OsZIP10* to identify the allelic variation. The molecular markers tagged with the particular trait are used in selection of elite genotypes with high grain Zn content.

Many studies have reported that the variation at the upstream region and introns of the gene also affects the gene expression and hence leads to the phenotypic variation. Lingle and Dyre (2004), studied the polymorphism in the promoter region (2000 bp) of the sucrose synthase-2 gene of *Saccharum* genotypes and found insertion-deletions (*InDels*) ranging in size from 233 to 247 bp followed by designing of PCR based marker based on Indel region. Hayashi et al. (2006) developed PCR-based *InDel* markers for nine blast resistance genes by using already reported sequence information on the candidate genes. Sumantha et al. (2014) revealed that nucleotide variations were present in monomorphic marker OsZIP5b on agarose which was found to be significantly associated with grain zinc content. Similarly, in the present study, we have targeted upstream sequence of reported candidate gene *OsZIP10* to study variation. We found nucleotide variation in upstream sequence from the monomorphic bands of *ZIP10up* which was found to be associated with grain zinc content and further developed size based gene tagged marker and validated in 68 diverse rice genotypes. The markers can be used for genotyping in segregating populations and predicts the allelic status efficiently in many rice cultivars. The development of a gene tagged markers for this gene through the present study will be immensely helpful for enhancing the precision of selection in breeding programs. Several Quantitative Trait Loci (QTLs) for the grain micronutrient contents have been identified and mapped on different rice chromosomes using molecular markers but their refinement and genetic dissection are yet underway to truly understand the quantitative variation and genes contributing to the trait, which limits their effective utilization in MAS programs. *Indel* markers designed in the study can be validated in more germplasm lines and population. Newly developed gene tagged markers can be used as functional marker that can be useful for large scale screening and genotyping. Allele mining strategy can be employed to target other metal homeostasis related genes responsible for loading micronutrient content in grains or other low phenotypic variance trait.

Authors' contribution

Conceptualization of research (SM, MD, GC); Designing of the experiments (SM, MD, GC); Contribution of experimental materials (AKV, RS); Execution of field/lab experiments and data collection (SM, MD, AKV, RS); Analysis of data and interpretation (SM, GC); Preparation of manuscript (SM, GC).

Declaration

The authors declare no conflict of interest.

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